Vol. 129: 71–83, 2018
https://doi.org/10.3354/dao03233

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published June 19

Vibrio alginolyticus 16S–23S intergenic spacer region analysis, and PCR assay for identification of coral pathogenic strain XSBZ03

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ABSTRACT: *Porites andrewsi* white syndrome (PAWS), caused by *Vibrio alginolyticus* strains XSBZ03 and XSBZ14, poses a serious threat to corals in the South China Sea. To obtain a specific target against which to develop a rapid PCR detection method for the coral pathogenic strain XSBZ03, the 16S–23S rRNA gene intergenic spacer (IGS) region of 4 strains of *V. alginolyticus*, including the XSBZ03 and XSBZ14 strains, was amplified, sequenced and analyzed. Six types of IGS were found: IGS^{0}, IGS^{G}, IGS^{IA}, IGS^{AG}, IGS^{GLV}, and IGS^{GLAV}. IGS^{0}, IGS^{G}, IGS^{IA}, IGS^{AG} and IGS^{GLV} appeared to be the most prevalent forms in the 4 strains and the percentage identity range within each type was 91.4–100%, 89.3–98.5%, 83.0–99.8%, 91.5–95.6%, and 88.7–99.3%, respectively. IGS^{GLAV} was found only in the HN08155 strain, a causative agent of fish disease. IGS^{GLAV}, IGS^{GLV} and IGS^{AG} are reported here for the first time in *V. alginolyticus*. An IGS sequence specific to the XSBZ03 strain was identified following alignment of the homologous IGSs, and used to design strain-specific primers for its rapid identification by PCR. The results from PCR analysis suggest that the method is a rapid, practical, and reliable tool for the identification of the XSBZ03 strain in samples of isolated bacteria, as well as seawater and coral samples spiked with the bacterial strain. This is the first report of a rapid diagnostic assay for a causative agent of PAWS, based on PCR detection of a coral pathogen at the strain level. After applying this assay in coral transplantation, the survival rates of transplanted corals were significantly increased. This diagnostic assay should aid with both the elucidation of the cause of the disease, and transplantation of PAWS-free *P. andrewsi* in the South China Sea.

KEY WORDS: *Porites andrewsi* white syndrome · *Vibrio alginolyticus* · Intergenic spacer · IGS · Strain-specific primers · Diagnostic protocol

INTRODUCTION

Coral reefs worldwide have declined in recent years, particularly South China Sea reefs, Caribbean reefs, and Indo-Pacific reefs (Gardner et al. 2003, Bruno & Selig 2007, Xie et al. 2013). Coral diseases are one of the most serious hazards for the reefs (Rosenberg & Ben-Haim 2002, Rosenberg et al. 2007, Bourne et al. 2009). For example, severe coral diseases, such as *Montipora* white syndrome (WS), a tissue loss disease found in corals throughout the Hawaiian archipelago (Ushijima et al. 2012), white

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spot disease, coral black disease, yellow inflammatory-like syndrome, pink disease, and brown band disease occurring in some stony corals in the South China Sea, have been reported consistently in recent years (Huang et al. 2006, Qiu et al. 2010, Shi et al. 2012, Zhu et al. 2012). Despite the serious threat posed to the reef ecosystems, studies of coral disease etiologies and transmission dynamics have been frustrated by the inability to determine etiological agents for most of the observed diseases and there are few convenient diagnostic tools. In the South China Sea, *Porites andrewsi* is the dominant coral species in most coral reefs. Two *Vibrio alginolyticus* strains, XSBZ03 and XSBZ14, were isolated from the Qilianyu Islands (a subgroup of Xuande Atoll in the Xisha Archipelago) and determined to be the causative agents of *P. andrewsi* white syndrome (PAWS) in 2011 (Xie et al. 2013). The identification of pathogen-specific targets and development of subsequent rapid diagnostic methods are urgently needed.

The 16S–23S ribosomal rRNA gene intergenic spacer region (IGS), comprising tRNA genes and non-coding regions, lies between the 16S and 23S ribosomal DNA genes. As they are largely nonfunctional, IGS regions are under less evolutionary pressure, which results in their evolutionary rate being 10 times faster than that of 16S rDNA (Gürtler & Stanisich 1996). IGS regions, having a higher percentage of mutations compared to 16S rDNA and 23S rDNA (Barry et al. 1991, Gürtler & Stanisich 1996, García-Martínez et al. 1999), accumulate abundant taxonomical information (Kilian et al. 1989). Accordingly, they have been one of the most popular targets for bacterial taxonomy (Kilian et al. 1989, Maeda et al. 2000, Blaiotta et al. 2002). IGSs have also been widely used for the rapid identification of subspecies, including algae (Tan et al. 2010), fungi (Biondi et al. 2003, Wrent et al. 2010), mycoplasma (Takahashi-Omoe et al. 2004), and some bacteria, such as probiotic *Lactobacillus* species (Kwon et al. 2004), *Lactococcus* species (Blaiotta et al. 2002), *Streptococcal* species (Hassan et al. 2003), *Fusarium* species (Konstantinova & Yli-Mattila 2004), *V. parahaemolyticus* (Deng et al. 2007), *V. cholera* (Chun et al. 1999), and *V. mimicus* (Chun et al. 1999). Furthermore, analysis of the sequence variation of IGSs in many species, such as *V. parahaemolyticus*, *V. cholera*, and *V. mimicus* (Chun et al. 1999, Maeda et al. 2000, Hassan et al. 2003), using PCR-restriction fragment length polymorphism (RFLP) (Biondi et al. 2003, Konstantinova & Yli-Mattila 2004, Wrent et al. 2010) has shown several well-supported intra-species (strains or serotypes) clades with high-bootstrap values (Konstantinova & Yli-Mattila 2004). Hence, IGSs can be sufficiently variable to be used as the target to establish a PCR method for the specific identification of a certain strain.

In this study, IGSs of the *V. alginolyticus* strains XSBZ03, XSBZ14, HN08155, and ATCC 33787 were amplified by PCR, cloned, sequenced, and analyzed. An IGS region of strain XSBZ03 was identified as a target for specific PCR primers. The application of the PCR assay demonstrated that it could specifically amplify strain XSBZ03 among *Vibrio* strains or from environmental samples. This is the first report of a rapid diagnostic tool for the *V. alginolyticus* strain identified as the causative agent of PAWS. The assay should prove helpful in diagnosing PAWS and in the identification of PAWS-free *P. andrewsi* for transplantation in the South China Sea.

MATERIALS AND METHODS

Bacterial strains

The coral pathogenic *Vibrio alginolyticus* strains XSBZ03 and XSBZ14, which cause PAWS, were obtained from diseased bleaching corals in the Xisha Archipelago, South China Sea. In addition, 10 *Vibrio* spp. standard reference strains were obtained from the American Type Culture Collection (ATCC) (Table 1). A further 94 *Vibrio* spp. strains were collected from corals in the Xisha Archipelago (n = 11), marine rearing systems (n = 24) or diseased fish or shrimp (n = 59). These consisted of *V. parahaemolyticus* (2 strains), *V. rotifers* (1 strain), *V. harveyi* (38 strains) and *V. alginolyticus* (53 strains) (Table 1). They were all identified by methods reported previously (Xie et al. 2005, Cano-Gomez et al. 2009).

Culture media and DNA extraction

All of the *Vibrio* strains were isolated on TCBS (thiosulfate–citrate–bile salts–sucrose) agar culture medium. Colonies were removed from the TCBS agar and were inoculated at 30°C for 24 h in 5 ml marine broth 2216E (Xie et al. 2013) with constant shaking. Genomic DNA was extracted from 3 ml overnight cultures using a Bacterial Genomic DNA Extraction Kit Ver.3.0 (TaKaRa). Extracted DNA was eluted with 50 µl TE buffer and stored at –20°C until use.

Table 1. Reference strains examined in this study and the results of their detection with the specific PCR primers designed against *Vibrio alginolyticus* strain XSBZ03. (–): negative; (+): positive

Strain	Species	Source	PCR product
XSBZ03	*V. alginolyticus*	Diseased *Porites andrewsi*, Xisha Archipelago	+
XSBZ14	*V. alginolyticus*	Diseased *Porites andrewsi*, Xisha Archipelago	–
ATCC 33787	*V. alginolyticus*	–	–
ATCC 17802	*V. parahaemolyticus*	–	–
ATCC 33813	*V. furnissii*	–	–
ATCC 33788	*V. natriegens*	–	–
ATCC 27562	*V. vulnificus*	–	–
ATCC 33653	*V. mimicus*	–	–
ATCC 33810	*V. fluvialis*	–	–
MCCC 1A00057	*V. ichthyoenteri*	–	–
MCCC 1H00002	*V. aestuarianus*	–	–
MCCC 1H00014	*V. gazogenes*	–	–
XSE386	*V. parahaemolyticus*	Healthy corals, Xisha Archipelago	–
HN076	*V. rotiferianus*	Healthy corals, Xisha Archipelago	–
XSA056	*V. parahaemolyticus*	Healthy corals, Xisha Archipelago	–
HN08155	*V. alginolyticus*	Diseased *Epinephelus coioides*, Xincun Bay in Lingshui City	–
HN08335	*V. alginolyticus*	Diseased *Epinephelus coioides*, Xincun Bay in Lingshui City	–
HN08801	*V. alginolyticus*	Rearing water, Hainan	–
HN08307	*V. alginolyticus*	Rearing water, Xincun Bay in Lingshui City	–
HN08805	*V. alginolyticus*	Rearing water, Hainan,	–
HN08810	*V. alginolyticus*	Rearing water, Hainan	–
HN08306	*V. alginolyticus*	Seawater, Hainan	–
HN07011	*V. alginolyticus*	Rearing water, Hainan	–
HN07002	*V. alginolyticus*	Seawater, Haikou, Hainan	–
HN07006	*V. alginolyticus*	Diseased *Lutianus argentimaculatus*, Hainan	–
HN07010	*V. alginolyticus*	Rearing water, Hainan	–
HN08202	*V. alginolyticus*	Rearing water, Hainan	–
HN07014	*V. alginolyticus*	Rearing water, Hainan	–
HN07005	*V. alginolyticus*	Seawater, Haikou, Hainan	–
HN08806	*V. alginolyticus*	Rearing water, Hainan	–
HN08808	*V. alginolyticus*	Diseased *Lutianus argentimaculatus*, Hainan	–
HN08201	*V. alginolyticus*	Rearing water, Hainan	–
TG06003	*V. alginolyticus*	Seawater, Hainan	–
XSE381	*V. alginolyticus*	Healthy corals, Xisha Archipelago	–
HN08803	*V. alginolyticus*	Rearing water, Hainan	–
HN08303	*V. alginolyticus*	Rearing water, Hainan	–
HN08203	*V. alginolyticus*	Rearing water, Hainan	–
HN07009	*V. alginolyticus*	Seawater, Hainan	–
HN08305	*V. alginolyticus*	Rearing water, Hainan	–
HN07008	*V. alginolyticus*	Rearing water, Hainan	–
ZJ51	*V. alginolyticus*	Rearing water, Zhanjiang, Hainan	–
HN08156	*V. alginolyticus*	Pearl River Fisheries Research Institute, Guangzhou, Guangdong	–
HN901	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN601	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN802	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN1001	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN703	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
NA25	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
E259	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
E333	*V. alginolyticus*	Healthy corals, Xisha Archipelago	–
22	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
20	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
12	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
15	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
10	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
21	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
4	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
27	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–
24	*V. alginolyticus*	Diseased *Litopeaneus vannamei*, Wanning	–

(Table continued on next page)

Table 1 (continued)

Strain	Species	Source	PCR product
E001	*V. alginolyticus*	Healthy corals, Xisha Archipelago	–
HN8813	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN8305	*V. alginolyticus*	Natural seawater, Haikou	–
HN08304	*V. alginolyticus*	Rearing water, Haikou	–
HN7014	*V. alginolyticus*	Healthy coral, Xisha Archipelago	–
HN7010	*V. alginolyticus*	Healthy coral, Xisha Archipelago	–
HN8809	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN08811	*V. alginolyticus*	Diseased *Epinephelus coioides*, Haikou	–
HN08807	*V. alginolyticus*	Natural seawater, Haikou	–
GDH11388	*V. harveyi*	Healthy coral, Xisha Archipelago	–
E399	*V. harveyi*	Stocked in Hainan University, Haikou	–
NS131241	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
NS131531	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
NS131651	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
NS131841	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
WC13D121	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
WC13D151	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
WC13D131	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
WC13DH11	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
WC13DH21	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
NS131341	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
WC13DH31	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
WC13H252	*V. harveyi*	Diseased *Lutianus argentimaculatus*, Wenchang	–
XC130452	*V. harveyi*	Diseased *Epinephelus coioides*, Lingshui	–
NS131251	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
NS131631	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
NS131851	*V. harveyi*	Diseased *Epinephelus coioides*, Sanya	–
WC13D231	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH1103	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH1104	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH1113	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
GDH11387	*V. harveyi*	Healthy coral, Xisha Archipelago	–
WC13D261	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH1101	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH1102	*V. harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH11013	*V. Harveyi*	Diseased *Lutjanus erythopterus*, Wenchang	–
HNH1106	*V. harveyi*	Diseased *Lutjanus erythopterus*, Haikou	–
HNH1107	*V. harveyi*	Diseased *Lutjanus erythopterus*, Haikou	–
HNH1108	*V. harveyi*	Diseased *Lutjanus erythopterus*, Haikou	–
HNH1109	*V. harveyi*	Diseased *Lutjanus erythopterus*, Haikou	–
HNH1111	*V. harveyi*	Diseased *Lutjanus erythopterus*, Haikou	–
NS131051	*V. harveyi*	Diseased *Lutjanus erythopterus*, Sanya	–
XC130351	*V. harveyi*	Diseased *Lutjanus erythopterus*, Lingshui	–
HNH11003	*V. harveyi*	Diseased *Lutjanus sanguineus*, Haikou	–
GDH11389	*V. harveyi*	Healthy corals, Xisha Archipelago	–
NS131451	*V. harveyi*	Diseased *Lutjanus erythopterus*, Sanya	–
NS131751	*V. harveyi*	Diseased *Lutjanus erythopterus*, Sanya	–

PCR primers for IGS amplification

Primers were designed to amplify the IGS of *V. alginolyticus*. The forward primer, VINTF (5′-TGG GGT GAA GTC GTA ACA AGG-3′), was designed from the 16S rDNA corresponding to positions 1485 to 1505 of *Escherichia coli* (Vold 1985; GenBank accession number: J01859.1). The reverse primer, VINTR (5′-TCC TTC ATC GCC TCT GAC TG-3′), was designed based on positions 37 to 56 of the 23S rDNA of *E. coli* (Maeda et al. 2000; GenBank accession number: AE014075.1).

PCR and cloning

The DNA of *V. alginolyticus* strains XSBZ03, XSBZ14, ATCC 33787, and HN08155 was subjected

to PCR amplification in a total volume of 25 µl containing 12.5 µl of Premix Taq™ (TaKaRa Taq™ Version 2.0), 1 µl of bacterial genomic DNA solution, 1 µl (each) of primers VINTF and VINTR, and 9.5 µl of ddH_2O. The thermal cycling parameters were as follows: 95°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and an additional extension at 72°C for 10 min. The presence and size of PCR products were determined by 1% agarose gel electrophoresis. The products of successful amplification reactions (which contained a number of different sized PCR products within each reaction) were purified using the DNA Fragment Purification Kit (TaKaRa MiniBEST DNA Fragment Purification Kit Ver. 4.0) and were inserted into the pMDTM19-T vector (TaKaRa pMDTM 19-T Vector Cloning Kit) before being introduced into *E. coli* JM109. The size of the inserted PCR products in vector-transformed *E. coli* colonies was determined by 1% agarose gel electrophoresis of PCR products amplified from colonies with M13 primers (TaKaRa), a pair of universal primers which bind on either side of the vector insertion site.

Sequencing of IGS and data analysis

Positive clones, representing the different sized IGS insertions, were randomly selected to be sequenced automatically using an ABI Prism 3730 XL DNA Analyzer System (Applied Biosystems). All of the sequences were submitted to GenBank. Characteristics of the sequences were examined using GenBank BLAST and tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/) (Lowe & Chan 2016). To identify strain-specific targets, Editseq, ClustalX2, and MegAlign were used to analyze the sequence. DNAman was used to determine identity. All the parameters were set to default.

PCR primer design for XSBZ03 detection

The XSBZ03-specific PCR primers (BZRDF/BZRDR) were designed using an IGS sequence region specific to the strain (accession number: KT779052). The target region was selected based on the alignment of the IGSs of the 4 *V. alginolyticus* strains XSBZ03, XSBZ14, HN08155, and ATCC 33787. The alignment results demonstrated that there was an insertion of ACA TTG TTT CTG CTT TTA GCA AAA CAA CCT A between sites 238 and 269 of IGS^{AG} from strain XSBZ03 (697 bp). This region was targeted for the design of a specific reverse primer, BZRDR (5′-ACG GGA CAC TGT AGG TTG TT-3′) (see Fig. 2). The forward primer, BZRDF (5′-ACG GTT TAG ATT TCA GAG CA-3′), was designed based on positions 98 to 117 of IGS^{AG} from strain XSBZ03 (697 bp). The primers were checked for specificity against the GenBank nucleotide database using BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/, parameters: Database = nr; Organism = bacteria(taxid:2)) and analyzed with the Ribosomal Database Project's (RDP) Probe Match (https://rdp.cme.msu.edu/, default parameters). They were synthesized by the Beijing Genomics Institute (BGI).

Checking on the specificity of the designed primers by PCR

PCR amplification was performed using extracted genomic DNA from each of the *Vibrio* spp. strains as a template and was optimized in a 25 µl reaction mixture consisting of 12.5 µl of Mix (TaKaRa), 1 µl of bacterial genomic DNA solution, 1 µl (each) of primers BZRDF and BZRDR, and 9.5 µl of ddH_2O. The thermal cycling parameters were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 1 min, and a terminal extension step of 72°C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis to confirm the presence of the amplicon. Next, the amplicon was separated from the agarose gel using an UltraCleanTM15 DNA Purification kit (MO BIO Laboratories) and was cloned into the pMDTM 19-T vector (TaKaRa pMDTM 19-T Vector Cloning Kit). The inserted PCR products were sequenced by BGI. The specificity of the designed primers was confirmed since the expected amplicon was only produced from the XSBZ03 genome.

Sensitivity of the PCR detection of strain XSBZ03

In order to determine the detection limits of the XSBZ03 PCR assay in a purified bacterial suspension or in seawater, a culture suspension of the strain XSBZ03 was 10-fold serially diluted from 10^8 CFU ml^{-1} to extinction with saline solution (2% NaCl) or with seawater from 3 different sources (each regarded as a sample: S1, S2, S3). The pH and salinity of the seawater were assayed directly by a PHS-25CW pH meter (Bante) and a SALscan20 salinity meter (Bante). The total number of bacteria, or vibrios only, in the seawater was assessed respectively on 2216E or TCBS agar, incubated at 30°C until suffi-

cient colony growth, followed by determination of the CFU (see Table 3). To confirm the concentration of strain XSBZ03, 100 µl of the 10-fold dilutions were spread onto 2216E agar, incubated at 30°C until sufficient colony growth, and the CFU were determined. The samples were used as templates to determine the analytical sensitivity of the PCR assay for strain XSBZ03. PCRs were carried out using 1 µl of the diluted suspensions (in saline solution or in the seawater samples) as template and 5 µl of the resulting products were analyzed by 1% agarose gel electrophoresis at a constant voltage of 5 V cm^{-1}, to confirm the presence of the amplicon.

Simultaneously, to determine the analytical sensitivity limits of the PCR in the presence of *Porites andrewsi* nubbin material, 1 g of coral tissue was mixed with 1 ml (1.68×10^6 CFU) of strain XSBZ03 in different suspensions (in 2% NaCl saline solution, S1, S2, and S3). DNA was extracted from the 4 groups of mixed samples and resuspended in 100 µl TE buffer. The concentration was measured using a micro-volume spectrophotometer (MaestroNano). The extracted DNA was 10-fold serially diluted and 1 µl samples were used as template in PCR reactions as described previously.

Application of the PCR assay during coral transplantation

The coral transplantation experiments were carried out in 2014 and 2015. Healthy corals were sampled from 2 locations (see Table 4) and divided into small nubbins. DNA was extracted from 1 cm^2 of tissue from each nubbin as template for the XSBZ03 PCR assay to determine presence of the pathogenic strain. The nubbins were tied onto a plastic net fixed on the seabed (16° 28.5′ N, 111° 45.0′ E), where corals were almost extinct, and observed for at least 6 mo to calculate the survival rates.

RESULTS

IGS analysis of the 4 strains

For all 4 *Vibrio alginolyticus* strains, XSBZ03, XSBZ14, ATCC 33787, and HN08155, the IGS region was amplified with the primers VINTF and VINTR, and the PCR products were analyzed by agarose gel electrophoresis. Four visible bands of different sizes and intensities were obtained for each strain (Fig. 1). A total of 400 to 500 positive clones containing vectors with IGS inserts were analyzed by PCR for each of the 4 *V. alginolyticus* strains. These were then classified into 3 groups based on the size of the inserted IGS fragment. An approximate ratio of 3:3.5:1 was found for clones harboring inserted fragments of 400–500 bp (Group 1), 500–750 bp (Group 2), and 750–1000 bp (Group 3), respectively. For each strain, 30 different IGS clones (giving 12, 14, and 4 clones representing the respective size groups) were analyzed by DNA sequencing.

A total of 36 types (by sequence length) of IGS were identified among the 4 *Vibrio* strains, and their profiles and characteristics are summarized in Table 2. The IGSs were classified into 6 groups according to the number and type of tRNA gene(s) present in the sequence. The 6 groups were as follows: IGS^0, which contained no tRNA; IGS^G, coding for $tRNA^{Glu}$; IGS^{IA}, coding for $tRNA^{Ile}$ and $tRNA^{Ala}$; IGS^{AG}, coding for $tRNA^{Ala}$ and $tRNA^{Glu}$; IGS^{GLV}, coding for $tRNA^{Glu}$, $tRNA^{Lys}$, and $tRNA^{Val}$; and IGS^{GLAV}, including the cluster of $tRNA^{Glu}$, $tRNA^{Lys}$, $tRNA^{Ala}$, and $tRNA^{Val}$. IGS^0, IGS^G, IGS^{IA}, IGS^{AG}, and IGS^{GLV} appeared to be the most prevalent IGS forms found in the 4 strains, but IGS^{GLAV} was only found in strain HN08155 (accession number: KT808671). Further-

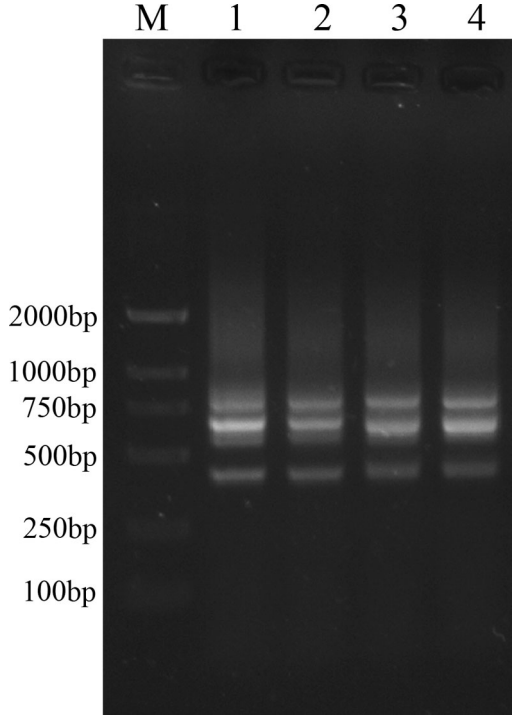

Fig. 1. Electrophoresis of the PCR-amplified 16S–23S rDNA intergenic spacers of 4 *Vibrio alginolyticus* strains. Lane M, molecular marker (DL2000 DNA Marker); Lane 1, ATCC 33787; Lane 2, XSBZ03; Lane 3, XSBZ14; Lane 4, HN08155

Table 2. Size and tRNA composition of the 16S–23S rRNA gene intergenic spacers (IGS) of 4 *Vibrio alginolyticus* strains. A total of 120 clones were analyzed by DNA sequencing and 3 clones failed to yield sequences

Type	tRNA genes contained	Identity within strain (%)	Length (bp)	No. of clones	Accession number
HN08155					
IGS^{0}	0	100	391	10	KT735056
IGS^{G}	Glu (UUC)	98.5	546	1	KT751230
			547	8	KT751231
IGS^{IA}	Ile (GAU), Ala (UGC)	84–95.8	640	5	KT751233
			642	1	KT751234
IGS^{AG}	Ala (GGC), Glu (UUC)	100	672	1	KT751232
IGS^{GLV}	Glu (UUC), Lys (UUU), Val (UAC)	100	784	3	KT779054
IGS^{GLAV}	Glu (UUC), Lys (UUU), Ala (UGC), Val (UAC)	100	842	1	KT808671
ATCC 33787					
IGS^{0}	0	100	420	12	KT779059
IGS^{G}	Glu (UUC)	100	580	4	KT779036
IGS^{IA}	Ile (GAU), Ala (UGC)	93.8–99.4	662	3	KT779040
			664	3	KT779041
			674	2	KT779042
IGS^{AG}	Ala (GGC), Glu (UUC)	93.8	673	2	KT779051
			666	1	KT779050
IGS^{GLV}	Glu (UUC), Lys (UUU), Val (UAC)	100	811	3	KT779055
XSBZ03					
IGS^{0}	0	96.7	420	9	KT779060
			421	4	KT779061
IGS^{G}	Glu (UUC)	100	573	3	KT779037
IGS^{IA}	Ile (GAU), Ala (UGC)	96.2–98.5	663	1	KT779043
			660	1	KT779044
			665	3	KT779045
			668	2	KT779046
IGS^{AG}	Ala (GGC), Glu (UUC)	100	697	2	KT779052
IGS^{GLV}	Glu (UUC), Lys (UUU), Val (UAC)	99.3	808	1	KT779056
			842	1	KT779057
XSBZ14					
IGS^{0}	0	92.6–99	419	5	KT779062
			429	3	KT779063
			628	2	KT779064
IGS^{G}	Glu (UUC)	89.3	573	3	KT779038
			666	1	KT779039
IGS^{IA}	Ile (GAU), Ala (UGC)	84.7–99.8	638	5	KT779047
			638	1	KT779048
			661	2	KT779049
IGS^{AG}	Ala (GGC), Glu (UUC)	100	637	3	KT779053
IGS^{GLV}	Glu (UUC), Lys (UUU), Val (UAC)	100	816	5	KT779058

more, the sizes of the same IGS varied both within the same strain (e.g. IGS^{IA} in strain XSBZ03 showed sizes of 660, 663, 665, and 668 bp), and among strains (Table 2).

An analysis of the 36 types of IGS isolated from the 4 strains of *V. alginolyticus* showed the following ranges of percentage identity: IGS^{0} 91.4–100%; IGS^{G}s 89.3–98.5%; IGS^{IA} 83.0–99.8%; IGS^{AG}s 91.5–95.6%; and IGS^{GLV} 88.7–99.3%. The minimal identity of heteromorphic IGSs was 54.3% between IGS^{GLV} from XSBZ03 (842 bp) (accession number: KT779057) and IGS^{G} from XSBZ14 (666 bp) (accession number: KT779039). Alignment of homologous IGSs showed that there were 3 main variations, consisting of base substitutions, deletions, and insertions of blocks of nucleotides. These variations were observed to occur more commonly at the regions which did not code for the tRNAs. Of course, mutations also occasionally occurred in the region coding for the tRNA. For instance, A was replaced by T at position 290 of IGS^{IA} (664 bp in length) from the *V. alginolyticus* strain ATCC 33787.

In summary, heteromorphic IGS sequence types varied in the absence or presence of tRNA gene(s)

and both heteromorphic and homologous IGS sequence types showed nucleotide variation as a result of the deletion, insertion or substitution of 1 or more bases and blocks of nucleotides. IGS^{GLAV}, IGS^{GLV}, and IGS^{AG} are reported here for the first time in *V. alginolyticus*.

Design of the XSBZ03-specific primers

Alignment of the IGS sequences from the *V. alginolyticus* strains and other *Vibrio* species demonstrated that there is an insertion of ACA TTG TTT CTG CTT TTA GCA AAA CAA CCT A between sites 238 and 269 of IGS^{AG} from strain XSBZ03 (697 bp) (accession number: KT779052), whereas this unique region was not present in the IGS^{AG}s from the other *Vibrio* species and strains compared. Hence, that region was targeted for the design of a specific reverse PCR primer, BZRDR (5′-ACG GGA CAC TGT AGG TTG TT-3′) (Fig. 2). The forward primer, BZRDF (5′-ACG GTT TAG ATT TCA GAG CA-3′), was designed based on positions 98 to 117. The size of the PCR product is 182 bp.

Specificity of the PCR method

PCR amplification was carried out on the 106 strains shown in Table 1, which represent the coral pathogen *V. alginolyticus* strains, 10 standard *Vibrio* spp. strains, and additional *Vibrio* spp. strains isolated from diseased shrimp, fish, their rearing water, and seawater from different locations. As shown in Table 1, the 182 bp-specific amplicon was detected only in strain XSBZ03. The amplicon was sequenced and confirmed to be identical to the partial sequence of IGS^{AG} from strain XSBZ03.

Sensitivity of the PCR method

The analytical limits of rapid detection (LORD) of the PCR assay for *V. alginolyticus* XSBZ03 in bacte-

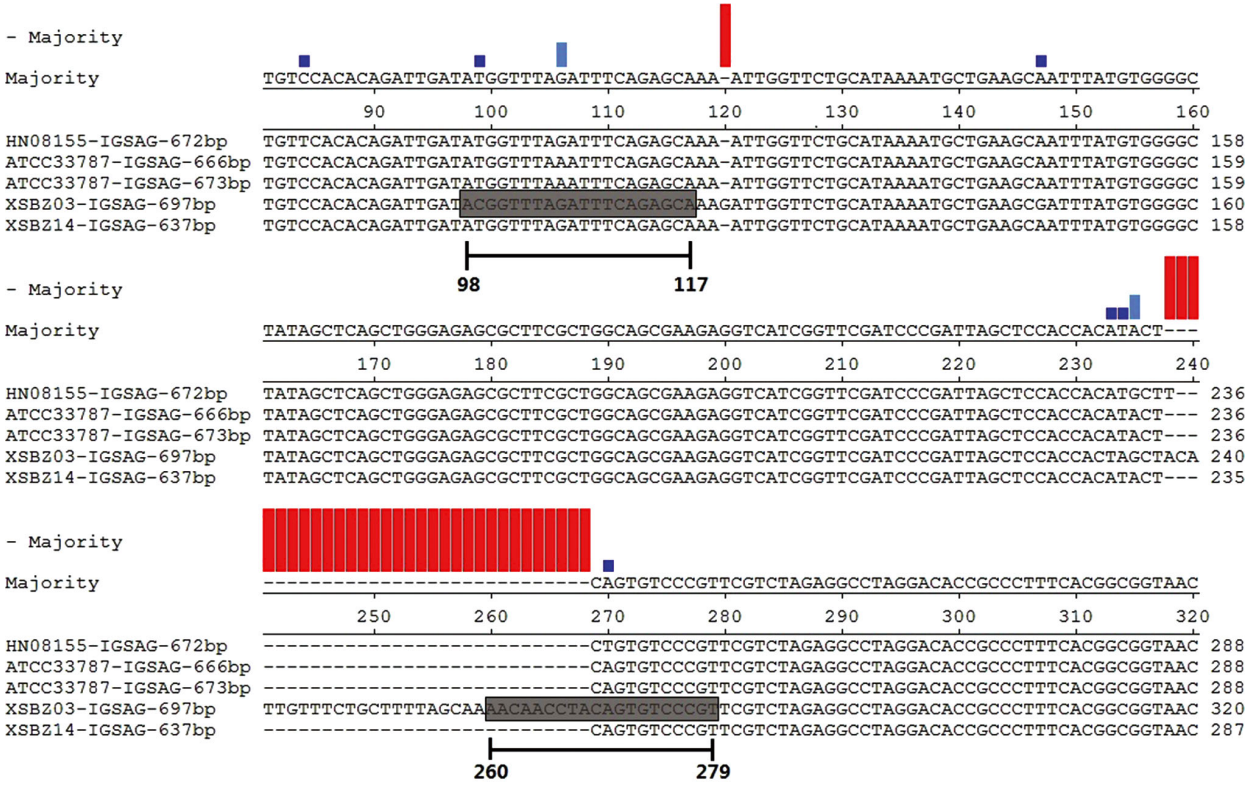

Fig. 2. Multiple alignment of the nucleotide sequences of IGS^{AG} from 4 *Vibrio alginolyticus* strains. The IGS^{AG} sequences are from strains HN08155 (672 bp, accession number: KT751232), ATCC 33787 (666 bp, accession number: KT779050), ATCC 33787 (673 bp, accession number: KT779051), XSBZ03 (697 bp, accession number: KT779052), and XSBZ14 (637 bp, accession number: KT779053). The sequence differences in the 5 IGS^{AG}s are marked by colored bars of different sizes (dark blue: minor variation; light blue: moderate variation; red: deletion). Broken lines indicate the gaps made for the alignment. The specific primers are highlighted in gray and underlined

rial suspension samples and extracted DNA samples are summarized in Table 3. For the bacteria in 2% NaCl saline solution, the LORD for 1 reaction was 2 cells (1.68×10^3 CFU ml^{-1} $\times 10^{-3}$ ml ≈ 2) (Fig. 3A). For the bacterial suspensions in natural seawater, the LORD for each reaction was 2 cells (1.68×10^3 CFU $ml^{-1} \times 10^{-3}$ ml ≈ 2), 17 cells (1.68×10^4 CFU $ml^{-1} \times 10^{-3}$ ml ≈ 17), and 17 cells (1.68×10^4 CFU $ml^{-1} \times 10^{-3}$ ml ≈ 17) for seawater samples S1, S2, and S3, respectively. For the coral samples mixed with bacteria, the LORD for each reaction was 1.68×10^4 CFU g^{-1} tissue (Fig. 3B). Thus, the PCR method established in the present study was sufficiently sensitive to detect 1.68×10^4 CFU of strain XSBZ03 in 1 ml liquid samples or in 1 g tissue samples, regardless of a whether bacterial suspension in seawater or bacterial DNA in a matrix of host DNA was used as the template.

Application of the PCR assay during coral transplantation

In 2014, the XSBZ03-specific PCR assay was not applied to detect the pathogen in healthy looking corals which were sampled to be transplanted. The survival rates of the 2 transplantation groups were 87.34% and 81.82%. In 2015, coral nubbins sampled from the same 2 locations (in Yongle Atoll in the Xisha Archipelago) were first divided into 2 groups. One group was used for transplantation directly while the other group was tested with the XSBZ03 PCR assay and the nubbins with positive PCR results were eliminated. A small portion (<10%) of the healthy looking corals tested positive for strain XSBZ03. After these had been discarded, the survival rates of the transplanted corals rose to 93.90% and 95.71%, compared to 82.95% and 87.18% for the untested corals (Table 4).

Table 3. The characteristics of 3 kinds of seawater and the limits of rapid detection (LORD) of spiked *Vibrio alginolyticus* strain XSBZ03 bacterial cells

Seawater (sources)	pH	Salinity	Total bacteria (CFU ml^{-1})	Total *Vibrio* spp. (CFU ml^{-1})	LORD (CFU ml^{-1})
Haikou Century Bridge	7.78	30	20 400	1370	1.68×10^3
Baishamen Beach	7.80	35	370	30	1.68×10^4
Laboratory aquaculture system	7.77	37	150	0	1.68×10^4

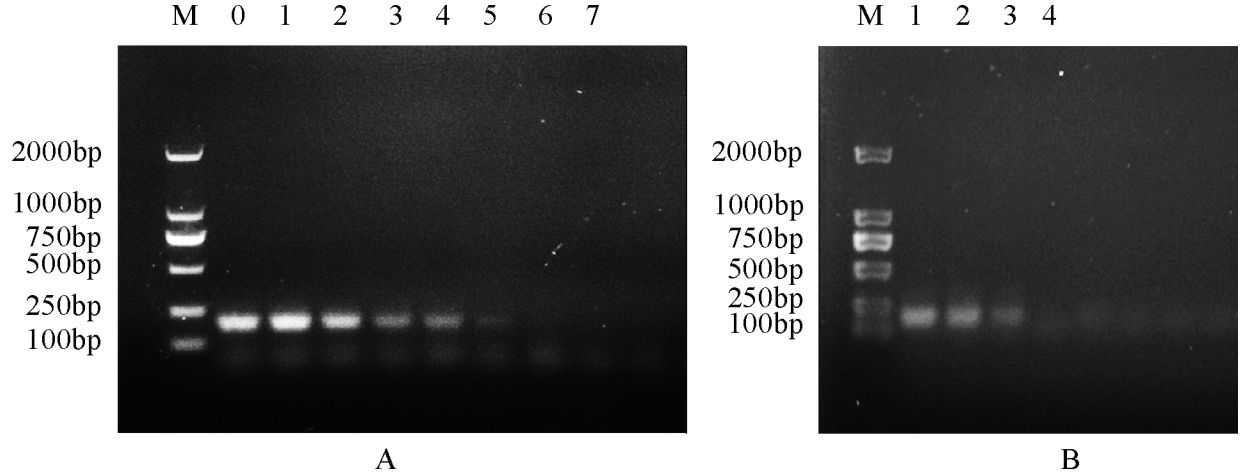

Fig. 3. Sensitivity of PCR with specific primers for the detection of coral pathogenic *Vibrio alginolyticus* strain XSBZ03. (A) PCR detection of dilutions of purified bacterial cells of strain XSBZ03 in saline solution (2% NaCl). Lane M, molecular marker (DL2000 DNA Maker); Lane 0, 1.68×10^8 CFU ml^{-1}; Lane 1, 1.68×10^7 CFU ml^{-1}; Lane 2, 1.68×10^6 CFU ml^{-1}; Lane 3, 1.68×10^5 CFU ml^{-1}; Lane 4, 1.68×10^4 CFU ml^{-1}; Lane 5, 1.68×10^3 CFU ml^{-1}; Lane 6, 1.68×10^2 CFU ml^{-1}; Lane 7, 1.68×10^1 CFU ml^{-1}. (B) PCR detection of DNA extracted from coral *Porites andrewsi* tissue mixed with strain XSBZ03. Lane M, molecular marker (DL2000 DNA Maker); Lane 1, 152 ng μl^{-1}; Lane 2, 15.2 ng μl^{-1}; Lane 3, 1.52 ng μl^{-1}; Lane 4, 0.152 ng μl^{-1} (corresponding samples contained 1.68×10^6, 1.68×10^5, 1.68×10^4, and 1.68×10^3 CFU g^{-1} tissue, respectively)

Table 4. Survival rates of transplanted *Porites andrewsi*. /: PCR assay not applied

Sampling site	Sampled nubbins	Results of PCR detection		Transplanted nubbins	Surviving nubbins	Survival rates (%)
		Positive nubbins	Negative nubbins			
2014						
16° 31.2′ N, 111° 45.8′ E	158	/	/	158	138	87.34
16° 28.6′ N, 111° 45.1′ E	132	/	/	132	108	81.82
2015						
16° 31.2′ N, 111° 45.8′ E	88	/	/	88	73	82.95
	88	6	82	82	77	93.90
16° 28.6′ N, 111° 45.1′ E	78	/	/	78	68	87.18
	77	7	70	70	67	95.71

DISCUSSION

Coral reefs have the highest productivity and biodiversity amongst the marine ecosystems, despite being in a low nutrient environment (Roberts et al. 2006). By providing various excellent biological habitats conducive to reproduction and feeding (Paulay 1997), reefs support 10% of the world marine fisheries catch (Smith 1978). Regardless of their type, coral reefs are a natural structure, irreplaceable in protecting the shoreline (Moberg & Folke 1999), monitoring and indicating the degree of pollution (Howard & Brown 1984), and recording the history of the marine climate (Zhao 1996, Beck et al. 1997). Furthermore, coral reefs are crucial for the biogeochemical circulation of carbon, nitrogen, and other elements (Parsons 1979). However, various coral diseases, especially white syndrome (WS) (Glynn 1993, Ushijima et al. 2012, Jones et al. 2014), have resulted in the extinction of many coral reefs and the breakdown of the reef structure. Because corals belong to the Cnidaria, having a hollow body cavity exposed to the seawater, it has been very difficult to address the true cause of coral disease. Phage therapy and the addition of antagonistic bacteria have been suggested as methods for mitigating outbreaks of coral disease (Teplitski & Ritchie 2009). In view of the extensive cost and potential environmental risk of phage therapy and antagonistic bacteria addition, it is critical to identify the actual pathogen causing the disease. Additionally, coral transplantation is another method that can be used to save local coral reefs but requires a complete absence of coral pathogens on the donor corals and in the marine environment into which transplantation occurs. In other words, the success of both prevention strategies and coral transplantation depends absolutely on a rapid and reliable detection method for specific pathogens of known coral diseases.

Vibrio alginolyticus is widely distributed in tropical marine environments (Xie et al. 2005, Yeh et al. 2009). As part of the normal marine flora (Austin et al. 1995, Vandenberghe et al. 1998), it has been shown to act as a probiotic in reducing fish pathogens, such as *Aeromonas salmonicida*, *V. anguillarum*, and *V. ordalii* (Austin et al. 1995, Gomez-Gil et al. 2002). However, many reports have also proven that *V. alginolyticus* is an opportunistic pathogen for aquacultured fish, shrimp, and shellfish, as well as humans (George et al. 2005). Therefore, it is important that the detection of *V. alginolyticus* pathogens occurs at the strain level. In recent years, *Porites andrewsi*, one of the dominant stony corals, has been seriously damaged in the South China Sea by PAWS caused by *V. alginolyticus* (Xie et al. 2013). In this study, a rapid detection method for the *P. andrewsi* coral pathogenic agent, *V. alginolyticus* strain XSBZ03, was successfully established. This is the first report of the development of a diagnostic assay for an agent of *P. andrewsi* disease, although there has been an urgent need in the South China Sea (Pollock et al. 2011). Based on IGS sequences, which show greater strain variability compared to 16S rDNA, strain-specific primers for *V. alginolyticus* strain XSZB03 were designed that allowed the accurate detection of this coral pathogenic agent at the strain level, something that could not be achieved in previous studies where only 16S rDNA was targeted (Polson et al. 2008). Additionally, the method was proven to be highly sensitive when it was applied to detect *V. alginolyticus* in 2 types of artificial samples, including dilutions of the bacterial suspension with seawater from 3 distinctive sources and DNA samples from coral tissues mixed with bacteria. Thus, we predict that the method established in this study should be capable of detecting *V. alginolyticus* strain XSBZ03 in bacterial isolates, environmental samples, and coral tissues, with a LORD of 1.68×10^3 to $1.68 \times$

10^4 CFU ml^{-1} seawater or 1.68×10^4 CFU g^{-1} tissue The results also suggest that the salinity might change the sensitivity level while the background bacteria in the seawater do not influence the LORD. When the salinity was greater than 30 (3%), the LORD changed from 1.68×10^3 to 1.68×10^4 CFU ml^{-1}. When using tissue samples, the host DNA or DNA loss in the extraction process also reduced the sensitivity to 1.68×10^4 CFU g^{-1} tissue.

After applying the PCR assay to coral samples from the Xisha Archipelago, we also found that some healthy-looking coral samples produced positive results. This suggests that strain XSBZ03 has a wide distribution in the South China Sea. As it is a causative agent of PAWS, these infections might become lethal after transplantation of the corals to a new location. After elimination of the coral nubbins with positive PCR results, the survival rates of the transplanted corals increased compared to the control group, which suggests that the use of the PCR assay for detection of strain XSBZ03 improved the success rate of the coral transplantation.

The IGS, as a carrier of tRNA genes, is very important for growth, reproduction, and other vital bacterial processes (Harvey et al. 1988, Lan & Reeves 1998, Liu & Sanderson 1998, Deng et al. 2006). The IGSs of 10 *Vibrio* species, including *V. alginolyticus*, *V. cholerae*, and *V. parahaemolyticus*, have been reported thus far. Most of the reports have focused on the structural analysis of the IGS of 1 strain, or have elucidated the differences in IGS sequences among several *Vibrio* species (Chun et al. 1999, Maeda et al. 2000, Lee et al. 2002). Although the comparison of IGSs among *V. alginolyticus* strains has previously been reported, only 3 IGS types, IGS^{IA}, IGS^{G}, and IGS^{0}, have been described (Deng et al. 2006). In this report, 5 types of tRNA and 6 types of polymorphic IGSs were obtained. The 4 types of tRNA, $tRNA^{Glu}$, $tRNA^{Ala}$, $tRNA^{Lys}$, and $tRNA^{Val}$, were found to exist in different combinations in the different IGS types, but with only 1 copy of each. Interestingly, IGS^{AG}, IGS^{GLV}, and IGS^{GLAV} of *V. alginolyticus* are reported here for the first time, and IGS^{GLAV} was only found in strain HN08155, a fish pathogenic agent. Therefore, the IGSs of *V. alginolyticus* exhibited sufficient diversity to allow the design of strain-specific primers and the development of a quick detection method for a specific strain of this species, as presented above. tRNA influences the expression of various virulence traits (Piechaczek et al. 2000, Weil et al. 2006). The variations in IGSs, as the carrier of tRNA, may reflect differences between virulent and non-virulent strains.

In conclusion, we have successfully established a PCR method to detect the coral pathogenic *V. alginolyticus* strain XSBZ03 to aid in the detection of this disease agent, PAWS diagnosis, and the transplantation of PAWS-free *P. andrewsi* in the South China Sea. With the increasing reports of genome sequences and available software packages for comparative genome analysis, assays for the detection of the other *V. alginolyticus* strain pathogenic to corals, XSBZ14, should also be established successfully.

Acknowledgements. This study was supported financially by the National Natural Science Foundation of China (No. 41466002, No. 31060360, and No. 30760190), the Fok Ying-Tong Education Foundation for Young Teachers in the Higher Education Institutions of China (No. 121030), Major Science and Technology Project of Hainan Province (No. ZDZX2013014), and Natural Science Foundation of Hainan Province (No. 311031).

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Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: August 21, 2017; Accepted: April 5, 2018
Proofs received from author(s): June 8, 2018